

DEVELOPMENT AND VALIDATION OF A SOLID-PHASE RADIOIMMUNOASSAY FOR MEASURING PROGESTERONE AND TESTOSTERONE IN OCTOPUS GONAD EXTRACTS

Omar Hernando Avila-Poveda^{1*, 2, 4}, Ruben Cornelio Montes-Pérez²,
Francisco Benítez-Villalobos³ & Carlos Rosas⁴

ABSTRACT

Commercially available radioimmunoassays (RIA) with ¹²⁵I-labeled hormones were adapted, developed and validated to quantify progesterone and testosterone in gonad extracts of *Octopus maya*, considered an easily domesticated species with potential in aquaculture. Development of the RIAs was divided into four phases: (1) extraction of progesterone and testosterone from gonads, (2) preparation of gonad extracts for RIA, (3) standards preparation, and (4) RIA procedure. Hormones were extracted twice with 15 mL of diethyl-ether each time. The recovered hormone and the solvent effect on extraction were evaluated. RIAs were validated based on specificity, sensitivity, accuracy, precision and reproducibility. Serially diluted gonad extracts were parallel to the standard curves of each hormone, indicating specificity. Sensitivities for both hormones were less than 0.1 ng•g⁻¹, and did not change by more than 20% during the ¹²⁵I half-life. Accuracy was 102.25 ± 11.22% for progesterone and 81.62 ± 6.24% for testosterone. For both hormones, precision was close to 13% and reproducibility 17%. The precision/reproducibility ratio indicated a system with good stability. The amounts of the hormones recovered increased when the number of extractions was increased. Hormone concentrations also increased when the volume of diethyl-ether used per extraction was increased. The range of progesterone and testosterone concentrations quantified in this study is in agreement with the range found in other octopuses. Finally, the developed and validated RIAs meet the requirements for quantification of progesterone and testosterone in *Octopus* gonad extracts and show that it is possible to use ¹²⁵I-labeled hormone as well as the plastic coated-tubes from commercial RIA kits to analyse variations in hormone levels associated with gonad developmental stages and the reproductive cycle of octopuses, allowing exploration of their possible functions.

Key words: hormone, commercial RIA kits, specificity, sensitivity, accuracy, precision, reproducibility, *Octopus maya*.

INTRODUCTION

Steroids are widely distributed throughout the animal kingdom, where they act as regulators of numerous biochemical and physiological processes (Finch & Rose, 1995; Lafont, 2000; Hanoune, 2007; Köhler et al., 2007). Aquatic invertebrates are able to produce “vertebrate-type” sex steroid hormones with a distribution consistent with their known reproductive roles in

vertebrate reproduction, which supports the idea of the universality of steroidogenesis (Carreau & Drosowsky, 1977; Sandor, 1980; Lafont & Mathieu, 2007; Di Cristo et al., 2010; Treen et al., 2012). It is generally agreed that the hormonal control of reproduction in invertebrates is not as well documented as in vertebrates, and even less so for cephalopod molluscs (Lehoux & Sandor, 1970; Joosse, 1972; Lafont, 2000; Ketata et al., 2008; Kikuyama & Tsutsui, 2011).

¹Ecología Marina, División de Estudios de Posgrado, Universidad del Mar (UMAR), Campus Puerto Ángel, Distrito de San Pedro Pochutla, Puerto Ángel, Oaxaca, C. P. 70902, México.

²Laboratorio de Radioinmunoanálisis, Facultad de Medicina Veterinaria y Zootecnia “FMVZ”, Universidad Autónoma de Yucatán (UADY). Mérida, Yucatán, México.

³Instituto de Recursos, Universidad del Mar (UMAR), Campus Puerto Ángel, Distrito de San Pedro Pochutla, Puerto Ángel, Oaxaca, C. P. 70902, México.

⁴Unidad Multidisciplinaria de Docencia e Investigación (UMDI), Facultad de Ciencias, Universidad Nacional Autónoma de México (UNAM). Puerto de Abrigo S/N. Sisal, Yucatán, México.

*Corresponding author: oavila@colombia.com

The presence of vertebrate-type steroid hormones has been documented in some invertebrate groups (Lehoux & Sandor, 1970; Lafont, 1991), but mainly in molluscs and echinoderms (Janer & Porte, 2007; Köhler et al., 2007; Lafont & Mathieu, 2007; Gust et al., 2010). In cephalopods, the reports are virtually restricted to a single species: *Octopus vulgaris* Cuvier, 1797. In many instances, the identification of these sex steroids relies only on radioimmunoassay (RIA) techniques. In fact, the RIA technique was the pioneer in the field with octopus and provided the preliminary evidence for the presence of “vertebrate-type” sex steroid hormones (i.e., progesterone, testosterone and/or 17 β -estradiol) in the reproductive system and particularly in the gonads of species of octopuses such as *Eledone cirrhosa* (Lamarck, 1798) (Nikitina et al., 1977; Young, 1992), *Octopus mimus* Gould, 1852 (Olivares-Paz et al., 2003), and *Octopus vulgaris* (D’Aniello et al., 1996; Di Cosmo et al., 2001). Coupled with RIA, other highly specific techniques as fluorometry (Nikitina et al., 1977), spectrophotometry (Nikitina et al., 1977) and HPLC (D’Aniello et al., 1996) have been used on octopuses as confirmative proofs. However, such proofs and others (e.g., gas chromatography or liquid chromatography coupled with mass spectrometric detection) require sophisticated equipment (Janer-Gual, 2005; Janer & Porte, 2007; Gust et al., 2010).

During the pioneering RIA studies using tritium labelled tracers to estimate progesterone and testosterone concentrations in octopus species (e.g., D’Aniello et al., 1996; Di Cosmo et al., 2001), the authors did not provide direct evidence of the reliability and validity of their method; nor did they investigate/optimize the extraction procedure. It is therefore difficult to use their protocols as a standardized method in order to assess progesterone and testosterone levels in cephalopod gonads. Of course, the validity and use of RIA is not considered novel; but RIA are still normally used as monitoring techniques in programs of octopus reproduction (Larson & Anderson, 2010), and there has been a growing interest in standardizing steroid hormone measurements by this immunological method due to its low cost and minimal hazards. Nevertheless, evaluation of analytical methods to measure sex steroid hormones in cephalopods requires reliability and validation; all the more so since the commercial kits are designed to be used in humans and not for the species in question

(Abraham, 1975; Cekan, 1975, 1979; Moss et al., 1976; Hafs et al., 1977; IAEA, 1984; Ezan et al., 1991; Chard, 1995).

Our aim was thus to adapt, develop and validate a commercially available solid-phase radioimmunoassay with ¹²⁵I-labelled hormones, as well as to describe a protocol of hormone extraction in order to measure progesterone and testosterone concentrations in octopus gonad extracts.

MATERIALS AND METHODS

Chemical and Reagents

All reagents were of HPLC grade. Diethyl-ether (CHROMASOLV® $\geq 99.9\%$ inhibitor-free), sodium phosphate dibasic, anhydrous ($\geq 98\%$, Fluka®), sodium phosphate monobasic, anhydrous ($\geq 99.0\%$, Fluka®), testosterone (purity $\geq 99.0\%$, Fluka®), and progesterone (minimum 99.0%, Sigma®) were from Sigma-Aldrich, Inc., Mexico. Methyl alcohol (J. T. Baker®) was from Mallinckrodt Baker, Inc., Phillipsburg, New Jersey, USA. Progesterone (DSL-3900) and testosterone (DSL-4000) coated-tube radioimmunoassay kits were purchased from Diagnostic Systems Laboratories®, Inc. Webster, Texas, USA.

Experimental Octopuses

Mature male ($n = 15$) and female ($n = 22$) *Octopus maya* Voss & Solís-Ramírez, 1966 (300–700 g body weight) used for validation of the RIA analysis were caught from January to May 2008 on the continental shelf of the Yucatán Peninsula in front of Sisal Harbor (21°9’55”N, 90°1’50”W). The octopuses were transported 300 m inland to the UMDI-UNAM laboratory; then kept in individual flow-through 80-L tanks with aerated seawater for two days without food to avoid feeding interfering with the physiological condition of the animals (Rosas et al., 2011). One PVC tube (10 cm in diameter) per octopus was placed in each tank as a refuge. Before dissecting the gonad, octopuses were immersed in seawater at 5°C for 3 min (derived from Roper & Sweeney, 1983) to induce a reduction in their metabolism (narcotisation), in consideration of ethics (Mather & Anderson, 2007) and their welfare during manipulations (Moltschaniwskyj et al., 2007), and it was performed in this way to avoid possible interference by chemical anaesthetics with the subsequent

TABLE 1. Steroid levels as determined by radioimmunoassay in *Octopus sp.** Authors within each source are ordered according to year of publication. The extraction procedure of steroid hormones from each tissue was performed two times, except where indicated by the symbol †. Refer to table 5 for the amount of solvent and the number of extractions.

Species	Tissue	Gonad development stages	Progesterone	Testosterone	Sources
<i>Eledone cirrhosa</i>	Ovary	third stage “mature”**	8.8	-	(Nikitina et al., 1977)
<i>Eledone cirrhosa</i>	Hemolymph	vitellogenesis	< 0.0013	< 0.000069	(Young, 1992)§
<i>Octopus vulgaris</i>	Testis	***	~3.3	~5.3	(D’Aniello et al., 1996)
	Hemolymph	***	~0.3	~0.4	
<i>Octopus vulgaris</i>	Ovary	Previtellogenesis	~0.030 to 0.040	-	(Di Cosmo et al., 2001)†
		early vitellogenesis	~0.040 to 0.045	-	
		full vitellogenesis	~0.200	-	
		late vitellogenesis	~0.020 to 0.025	-	
	Hemolymph	-	undetected	-	
<i>Octopus mimus</i>	Testis	Immature-maturing	~6.5 to 7.0	~7.2 to 8.5	(Olivares-Paz et al., 2003)
		mature	~7.0 to 8.2	~8.5 to 9.5	
		spawning	~3.5	~5.0 to 5.5	
<i>Octopus maya</i>	Ovary	mature	0.90 to 2.22	0.08 to 0.14	This study ^a
	Testis	mature	0.80 to 1.60	0.05 to 0.22	
<i>Octopus maya</i>	Ovary	mature	4.42 to 10.11	0.28 to 0.56	This study ^b
	Testis	mature	3.96 to 6.57	0.30 to 0.67	

*Concentration standardized to ng•g⁻¹ or ng•mL⁻¹.
**Considered as mature according to Arkhipkin (1992) and Boyle & Chevis (1992).
***Author indicated only adult males.
~Approximate values obtained from figures of each source.
-No data.
§Author indicated that hormonal level has little variation during the development of oocytes into the ripe stage.
†Only one extraction was performed.
^aSolvent amount used was 15 mL of diethyl-ether.
^bSolvent amount used was 30 mL of diethyl ether.

hormonal extraction. Soon after narcotizing the octopus, the gonad was removed, frozen in liquid nitrogen, and stored at -40°C until hormonal extraction.

Octopus maturity was confirmed both macroscopically as well as microscopically. Macroscopically in males by the creamy-white testis and Needham’s sac filled with spermatophores; and in females by the very large ovary, occupying the whole posterior half of the mantle cavity, with longitudinally striated egg yolk. Microscopically, the mature stage was verified in the gonads (i.e., testis and ovary), ovidu-

cal gland and spermatophoric complex (Fig. 1), following the procedures of Avila-Poveda et al. (2009); and staining with the modified Crossmon’s trichrome method (i.e., Groat’s hematoxylin, erythrosin B-Orange G and trypan blue, as derived from Crossmon, 1937; Gray, 1954; Gutiérrez, 1967).

Extraction of Progesterone and Testosterone from Gonads

The method of D’Aniello et al. (1996) was followed with some adjustments (i.e., timing and

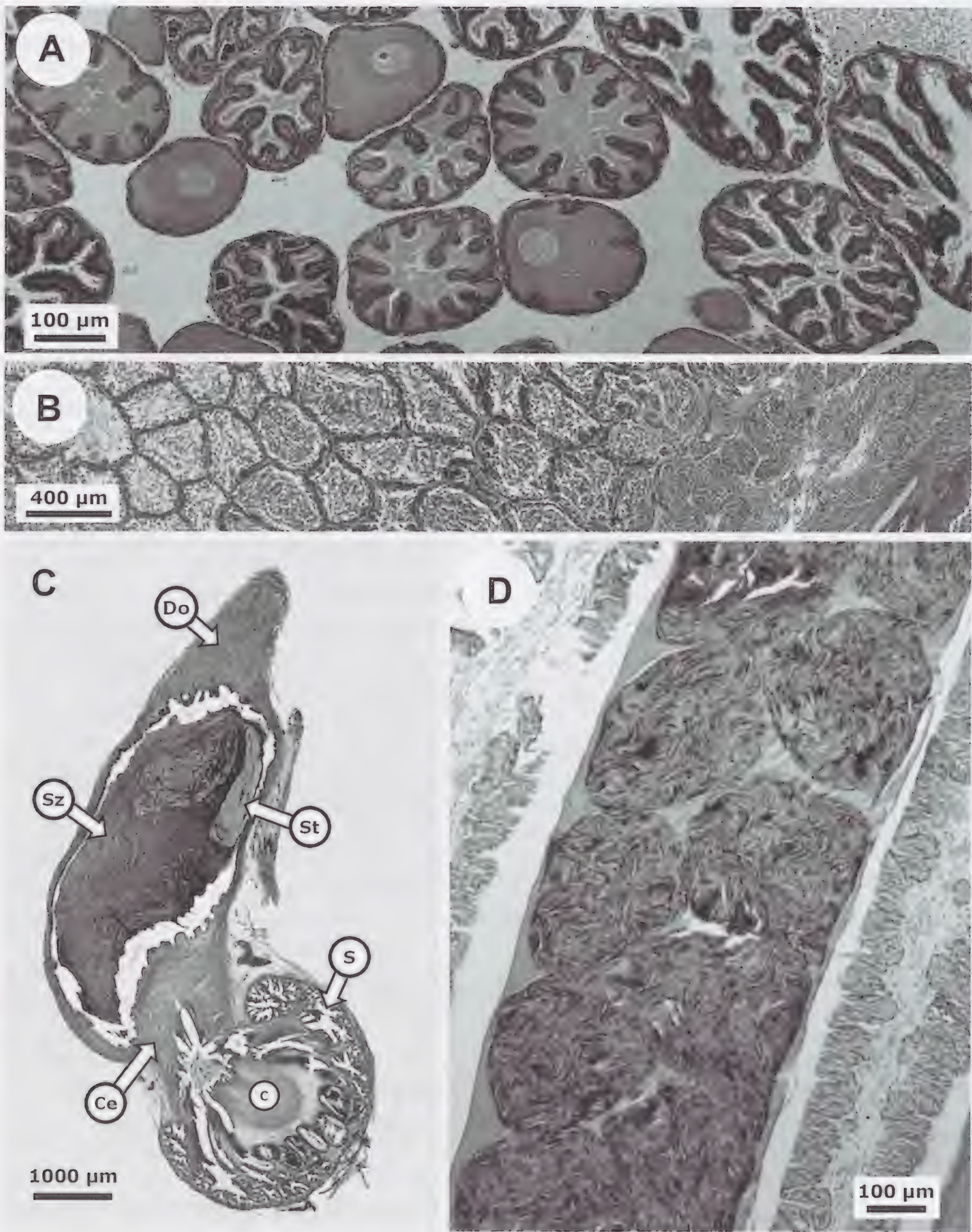


FIG. 1. Compound digital photomicrographs of histological sections of gonads and accessory glands of *Octopus maya* showing the physiological maturity and functional maturation (Arkhipkin, 1992) of the octopuses used in this study. A: Ovary at stage III, “mature”, with very large increase in oocyte diameters, primary follicle deeply embedded, and initial yolk development; B: Panoramic photomicrograph of the testis, showing Stage II (gametogenesis on the left side) to Stage III (mature on the right side and toward the accessory gland). In Stage II a mixture of spermatogonia, spermatocytes, spermatids, and spermatozoa is in the lumen of the seminiferous tubules, as well as a wide belt of spermatogonia surrounding the walls of the seminiferous tubules. In Stage III the lumen of the seminiferous tubules is filled with spermatozoa and surrounded by a narrow belt of spermatids; C: Transverse cross-section of the oviducal gland and distal oviduct of a female *Octopus maya* (373 g BW), showing the arrangement of the spermathecae (S) in several radial chambers that open into the central cavity (c) of the oviducal gland. Spermatozoa (Sz) were scarcely found within the spermathecae (S) but in a dense mass that also included a spermatophore (St) near the cervix (Ce) or neck of the oviducal gland, which corresponds to the lower narrow portion of the oviducal gland where it joins the distal oviduct (Do); D: Histological photomicrographs of the spermatophoric complex of a male *Octopus maya* (673 g BW) showing the arrangement of the spermatozoa constituting several dense masses within a spermatophore.

volume: CMFRI, 1983) which are subsequently described in detail: (1) 1 g of gonad (i.e., testis and/or ovary) was homogenized with 3 mL of distilled water for 3 min at a speed of 4000 rpm in a Tissue Homogenizing Systems Glas-Col®; (2) the homogenate was supplemented with 15 mL of diethyl-ether and vigorously mixed for 3 min (i.e., first extraction); (3) the mixture was set to rest for 45 min at 2–6°C; (4) the mixture was immersed in liquid nitrogen for 60 s and then the upper diethyl-ether phase was separated from the precipitate; (5) the extraction was repeated a second time from the precipitate obtained upon carrying out steps 2, 3 and 4; (6) the upper diethyl-ether phases were combined and evaporated in a hood with a switch-on airflow; and (7) the dry residue (i.e., gonad extract, GE) was stored at -20°C until measurement by RIA.

Preparation of Gonad Extracts for Radioimmunoassay

This procedure was developed according to Abraham (1975) and Abraham et al., (1977) and involved two additional major steps: (8) The stored GE was re-dissolved in 1000 µL methanol (M) and mixed for 3 min; (9) a 500 µL aliquot of the GE-M complex was transferred to a glass tube to which 500 µL of 0.1 M phosphate buffer solution (PBS), pH 7.0 was added and mixed for 3 min to form the GE-M-PBS complex that was used for RIA.

Standards Preparation

For both hormones, progesterone and testosterone, nine standards (in 0.1 M PBS, pH 7.0) were prepared by serial dilution and used at the following concentrations: 0.0, 0.0625, 0.125, 0.25, 0.5, 1.0, 2.0, 5.0 and 10.0 ng•mL⁻¹; covering the range of hormone levels reported from radioimmunoassay for *Octopus* spp. (Table 1).

Radioimmunoassay Procedure

All kit reagents (standards, labelled hormones, and coated tubes, including prepared gonad extracts) were at room temperature (~25°C) and mixed thoroughly to avoid foaming before use. All measurements were performed in duplicate and the duplicates were averaged into a single measurement. Solid-phase RIA for each hormone (progesterone and testosterone) was performed according to the manufacturer's

instructions that are described in detail subsequently: (1) each prepared standard and gonad extract was pipetted into the bottom of its corresponding coated-tube (50 µL for testosterone assay or 25 µL for progesterone assay); (2) 500 µL of ¹²⁵I-labeled hormone was immediately added to each coated-tube for the respective hormone assay; (3) each coated-tube was quickly vortexed; and (4) the tubes were then incubated for 60 min at 37°C in a water bath; (5) tubes were decanted, and allowed to drain until dry (around 2 min) before being read; (6) the radioactivity in each tube was quantified in a gamma-counter (Burnham-on-Crouch England) for 60 s.

Radioimmunoassay Analytical Validation

A curve-fitting (logit-log) technique was used, which applies logit (B/B₀) vs. log dose (Healy, 1972; Rodbard, 1974; Abraham et al., 1977; Govindarajulu, 2001). The reliability of the assays was validated based on their specificity, sensitivity, accuracy, precision and reproducibility.

Specificity or freedom from interference by substances other than the one to be measured was assessed by the parallelism test, assaying serially diluted ovary extracts (1:1, 1:2, 1:4 and 1:8) against the standard curve (Cekan, 1975, 1979; Hafs et al., 1977; IAEA, 1984), and plotting and testing the equality of the slopes (Zar, 2010). A lack of significant interaction between the serial dilutions and the standard curve indicates that the line slopes are similar. If the interaction is significant, then the slopes of the serially diluted sample and the standard curve are not parallel.

Practical and theoretical sensitivity of the assay was determined by calculating the smallest amount of hormone significantly different from zero at the 95% confidence limit (Abraham, 1975; Bedolla-Tovar et al., 1984; IAEA, 1984). For each hormone assay, cpm (counts per minute) of the zero standard were obtained from independent standard curves (n = 5 to 8) assayed after 6, 12, 28 and 46 days had elapsed in the half-life of ¹²⁵I:

$$\text{Practical Sensitivity} = [x - (2 * S.D.) / x] * 100$$

(IAEA, 1984)

$$\text{Theoretical Sensitivity} = 100 - [(t * C.V.) / \sqrt{n}]$$

(Bedolla-Tovar et al., 1984),

where *x*, *S.D.* and *C.V.* are the basic measurements of central tendency based on the counts per minute of the zero standard, *t* is Student's t-value tabulated to n-1 degrees of

freedom (95% confidence), and *n* is the number of zero standards measured.

Sensitivity changes throughout radioactive decay of ¹²⁵I were defined by changes in the values of the slopes of the standard curve assays on different days (Chase, 1979; Ezan et al., 1991; Alvarez-Cervera, 2002).

Assay accuracy denotes how close the results are to the true contents of unknown samples (Skelley et al., 1973; Abraham, 1975; Abraham et al., 1977; Cekan, 1975, 1979; IAEA, 1984), and was tested by adding a known quantity of standard to a GE-M-PBS complex (volume 1:2) from which all free steroids had first been removed with an active charcoal suspension of 0.6% (w/v) in PBS (Abraham et al., 1977). The accuracy of the RIA was tested by recovery experiments and was calculated as:

Recovery (%) = (Measured Value/Expected Value) x 100.

Precision of the assay was calculated by determining the intra-assay variability (CV) of duplicate measurements of the same sample in the same assay; while Reproducibility was determined using the inter-assay variability (CV) measuring the same sample in different assays (Skelley et al., 1973; Rodbard, 1974; Abraham, 1975; Cekan, 1975; IAEA, 1984; Ezan et al., 1991; Krotzky & Zeeh, 1995).

The intra and inter-CV were calculated as the standard deviation divided by the mean value. Generally, the inter-assay (between-assay) variance should be greater than the intra-assay (within-assay) variance; and the ratio of intra-assay to inter-assay variance provides an index of the temporal stability of the system (Rodbard, 1974).

Recovered Hormone

Previous pioneering studies with RIA in octopuses did not reveal the percentage of hormone recovered from gonads during extraction. The authors assumed that with one (Di Cosmo et al., 2001) or two extractions (Nikitina et al., 1977; D’Aniello et al., 1996; Olivares-Paz et al., 2003), the whole amount of hormone was recovered from the gonad. Then, the number of required extractions to obtain the entire hormone contained in the gonad was evaluated with a single mature ovary after making 2, 4, 6, 8 or 10 extractions, assuming that with the 10th extraction all the hormone (i.e., progesterone and testosterone) was extracted from the gonad.

Solvent Effect on Progesterone and Testosterone Extraction

Abraham (1975) indicated that the most common factor affecting the recovered concentration of hormone is the amount of solvent used during the extraction procedure; therefore, the highest quality should be used and in large amounts. Due to logistics, we used half (i.e., 15 mL of diethyl-ether) the amount of solvent indicated by D’Aniello (1996) for hormone extraction throughout this study. Even so, a complementary evaluation of the solvent effect on hormone extraction was made with a group of randomly selected samples; thus, the homogenates were extracted with 15 mL of diethyl-ether each time, but also with 30 mL of diethyl-ether in parallel (see methodology of extraction).

TABLE 2. Practical and theoretical sensitivities (ng·g⁻¹) of progesterone and testosterone assays throughout the radioactive decay of ¹²⁵I. (n = number of zero standard measured in duplicate, each derived from a standard curve).

Assay	Radioactive decay of ¹²⁵ I (days)			
	6	12	28	46
Progesterone (practical)	0.1308 (n = 6)	0.0572 (n = 6)	0.0952 (n = 4)	
Progesterone (theoretical)	0.0429 (n = 6)	0.0174 (n = 6)	0.0422 (n = 4)	
Testosterone (practical)	0.0688 (n = 5)	0.0032 (n = 4)		0.1012 (n = 8)
Testosterone (theoretical)	0.0175 (n = 5)	0.0011 (n = 4)		0.0170 (n = 8)

TABLE 3. Radioimmunoassay accuracy tested through recovery experiments (i.e., measured and expected values) for progesterone and testosterone from *Octopus maya* gonads.

Progesterone Expected Values = 3.33 ng•g ⁻¹ n = 15 samples		Testosterone Expected Values = 1.67 ng•g ⁻¹ n = 15 samples	
Measured Values	Recovery (%)	Measured Values	Recovery (%)
3.18	95.39	1.47	87.91
3.75	112.54	1.18	70.57
3.93	117.76	1.51	90.31
3.80	113.89	1.40	83.87
3.43	102.89	1.32	79.50
3.99	119.72	1.39	83.58
3.72	111.66	1.38	83.09
3.29	98.73	1.38	82.96
3.46	103.68	1.41	84.35
3.30	98.90	1.50	89.82
3.20	95.93	1.34	80.42
3.14	94.33	1.13	67.59
2.60	77.93	1.31	78.90
3.29	98.56	1.31	78.56
3.06	91.89	1.38	82.85
MEAN	102.25	MEAN	81.62
S.D.	11.22	S.D.	6.24

RESULTS

Radioimmunoassay Analytical Validation

Specificity. No significant differences were observed between the slope of the diluted sample and the standard curve for progesterone ($t_{0.05(2)10}$, $t_{cal} = 2.0943$, $P = 0.0626$) or for testosterone ($t_{0.05(2)10}$, $t_{cal} = 0.5540$, $P = 0.5917$), indicating that the serially diluted gonad extracts paralleled the standard curves of progesterone and testosterone. These results indicate linearity and specificity, i.e., that the antibody recognized the same structure immunologically in the unknown sample as in the standard curve.

Sensitivity. There were no significant differences ($P > 0.05$) between the practical and theoretical sensitivities of either hormone throughout the radioactive decay of ¹²⁵I, indicating that both determinations are consistent to show sensitivity. Sensitivities for both hormones were less than 0.1 ng•g⁻¹ (Table 2) and variations (i.e.,

values for the slopes of the standard curves) were not greater than 20% over 46 days of ¹²⁵I radioactive decay for both hormones (Fig. 2).

Accuracy. Table 3 shows the ranges of measured and expected values for progesterone and testosterone RIAs performed with two extractions. Briefly, the assay accuracy was 102.25 ± 11.22% for progesterone and 81.62 ± 6.24% for testosterone (n = 15).

Precision and Reproducibility. For both hormones, precision (intra-assay) was close to 13% and reproducibility (inter-assay) less than 17% (Table 4). The precision/reproducibility ratio was 75.39% for progesterone and 96.16% for testosterone, which indicates a good stability of the system.

Hormone Recovery

The fraction of hormone recovered with two extractions was 65% for progesterone and 83% for testosterone, but increased with a larger number of extractions (Fig. 3).

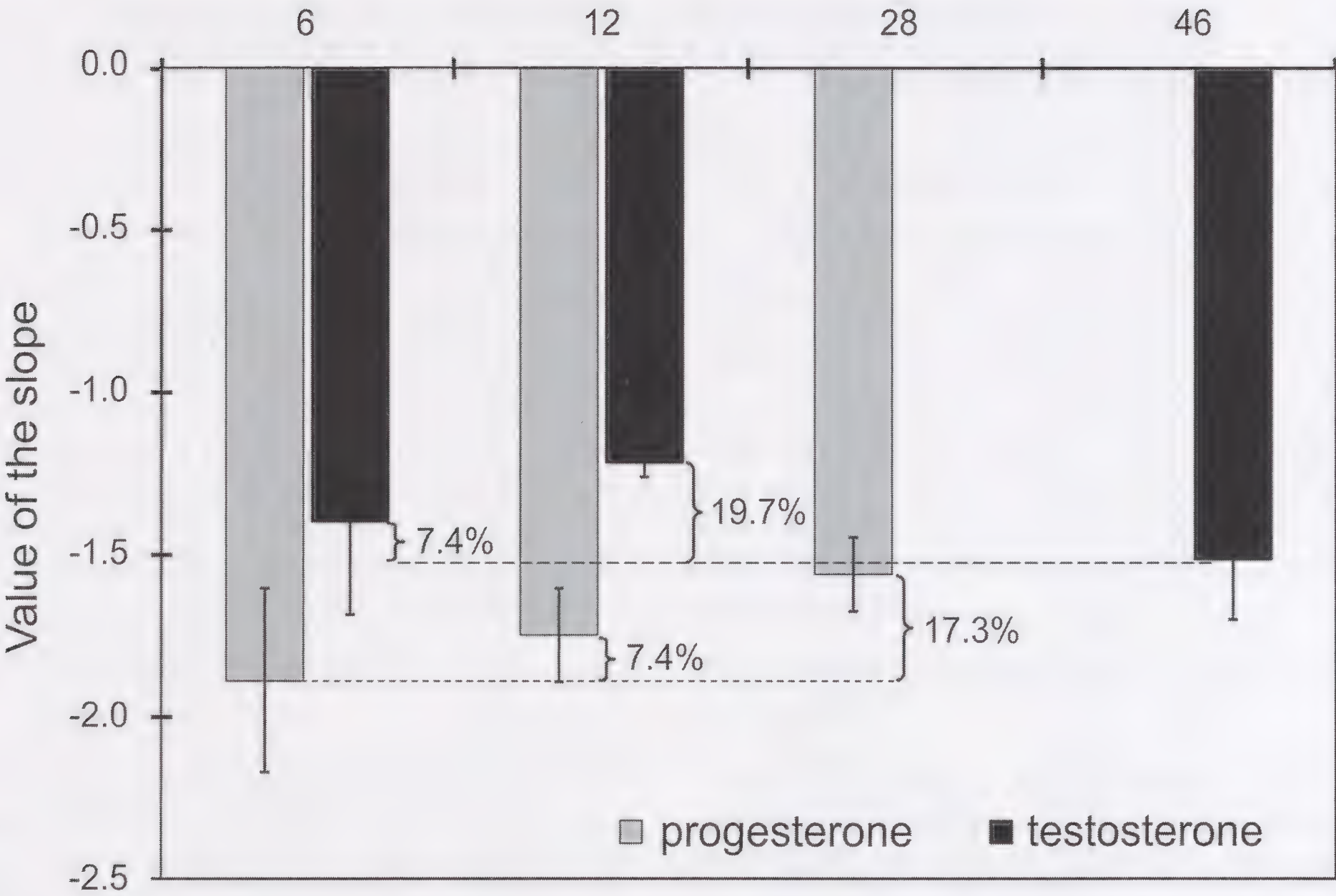


FIG. 2. Changes in progesterone and testosterone sensitivities with radioactive decay of ¹²⁵I.

TABLE 4. Intra- and inter-assay variability in tissue samples from *Octopus maya* in progesterone and testosterone RIA (sn = number of duplicate samples of the same RIA; rn = number of RIAs where the samples were assayed in duplicate; SD = standard deviation; CV = coefficient of variation).

Progesterone precision (Intra-assay variability)					Testosterone precision (Intra-assay variability)				
Sample	sn	mean (ng•g ⁻¹)	SD	CV (%)	Sample	sn	mean (ng•g ⁻¹)	SD	CV (%)
S1	2	1.64	0.10	6.03	S8	2	0.14	0.01	2.26
S2	2	1.11	0.27	24.71	S9	2	0.14	0.03	23.09
S3	2	1.48	0.13	8.89	S10	2	0.07	0.01	13.86
S4	3	1.83	0.13	6.94	S8	2	0.14	0.01	2.34
S5	2	1.50	0.27	18.22	S9	2	0.22	0.06	25.27
				12.96	S10	2	0.06	0.01	14.53
									13.56

Progesterone reproducibility (Inter-assay variability)					Testosterone reproducibility (Inter-assay variability)				
Sample	rn	mean (ng•g ⁻¹)	SD	CV (%)	Sample	rn	mean (ng•g ⁻¹)	SD	CV (%)
S4	3	2.17	0.35	16.09	S8	2	0.14	0.01	0.14
S5	3	1.78	0.40	22.49	S9	2	0.19	0.06	33.05
S6	3	0.99	0.11	11.52	S10	2	0.07	0.01	9.11
S7	2	0.64	0.12	18.65					14.10
				17.19					

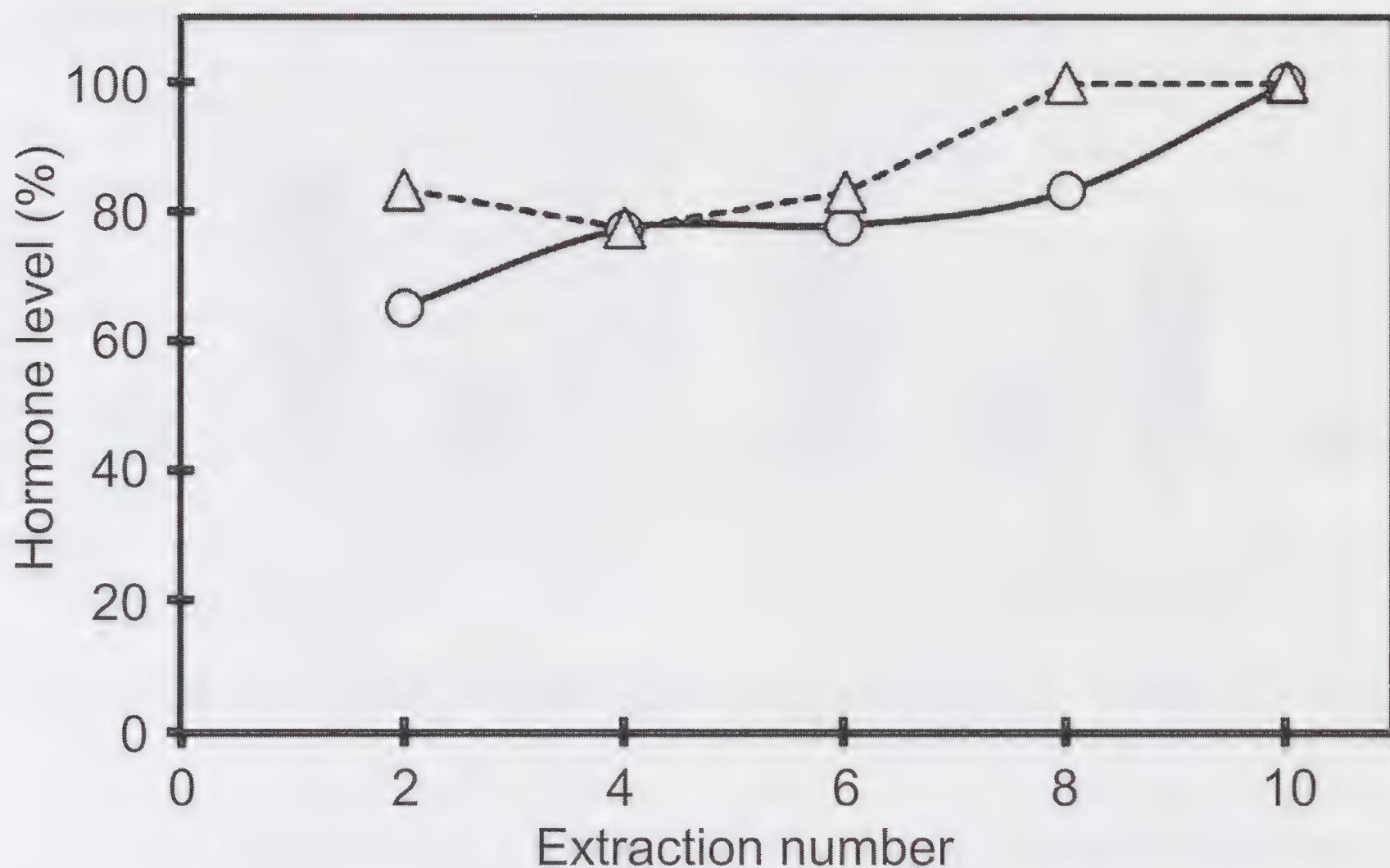


FIG. 3. Levels of recovered hormone in progesterone (○) and testosterone (Δ) radioimmunoassays according to the number of extractions made. It is assumed that with the 10th extraction all hormone in the tissue was recovered.

Solvent Effect on Progesterone and Testosterone Extraction

The concentrations of detected progesterone and testosterone following the extraction procedure with 15 mL of diethyl-ether per extraction were 4-fold lower, on average, than the levels of hormones detected following the same procedure but with 30 mL of diethyl-ether per extraction (Fig. 4, Table 5).

Progesterone and Testosterone Concentrations

Following extraction with 15 mL of diethyl-ether, the progesterone concentrations detected ranged from 0.90 to 2.22 ng•g⁻¹ in females and 0.80 to 1.60 ng•g⁻¹ in males; while, at a much lower concentration, the range of testosterone was 0.08 to 0.14 ng•g⁻¹ in females and 0.05 to 0.22 ng•g⁻¹ in males (Table 5).

TABLE 5. Amount of solvent used during the extraction procedure for RIA and the steroid level (i.e., progesterone and testosterone) reported. Authors within each source are ordered according to year of publication.

Tissue (g)	Solvent	Solvent amount	Extraction numbers	Progesterone (ng•g ⁻¹)	Testosterone (ng•g ⁻¹)	Sources
ovary, 5	acetone	No data	two	8.8	No data	(Nikitina et al., 1977)
testis, 1	diethyl-ether	30 mL	two	~3.3	~5.3	(D'Aniello et al., 1996)
ovary, --	methanol-water	70–30%	one	~0.020 to 0.200	No data	(Di Cosmo et al., 2001)
testis, --	diethyl-ether	5 volumes	two	~3.5 to 8.2	~5.0 to 9.5	(Olivares-Paz et al., 2003)
ovary, 1	diethyl-ether	15 mL	two	0.90 to 2.22	0.08 to 0.14	This study
ovary, 1	diethyl-ether	30 mL	two	4.42 to 10.11	0.28 to 0.56	This study
testis, 1	diethyl-ether	15 mL	two	0.80 to 1.60	0.05 to 0.22	This study
testis, 1	diethyl-ether	30 mL	two	3.96 to 6.57	0.30 to 0.67	This study

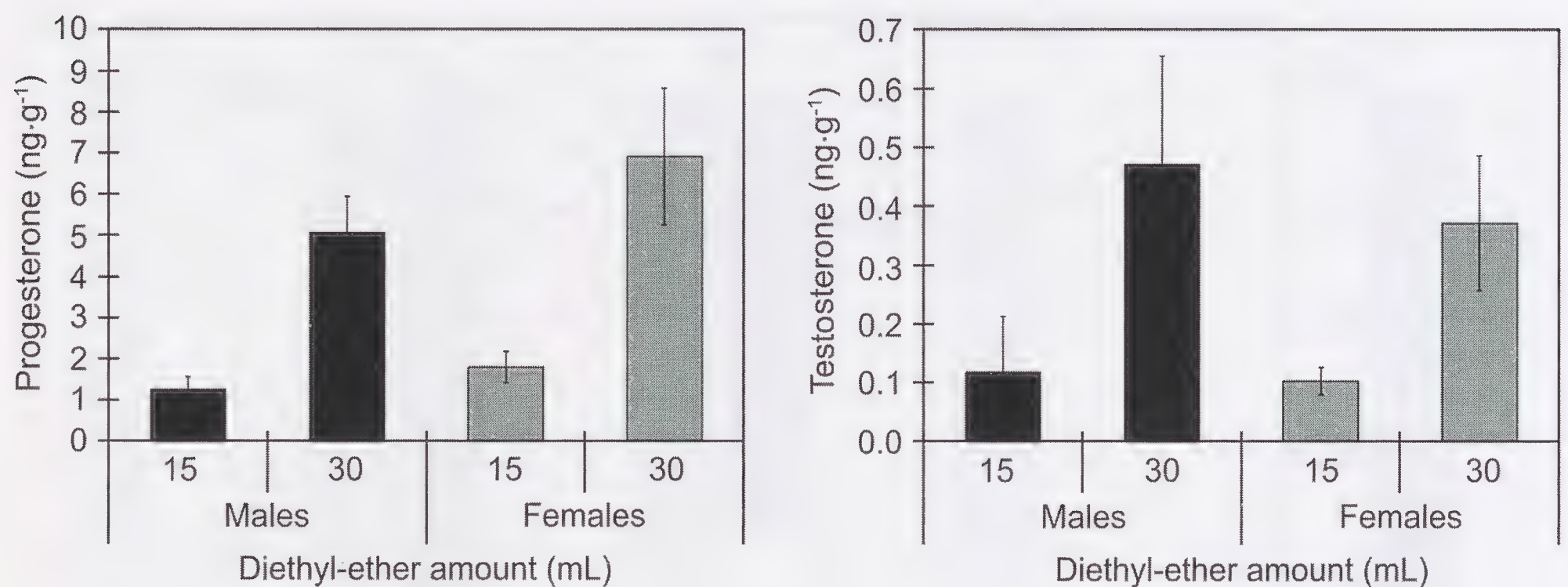


FIG. 4. Effect of the amount of diethyl-ether on the level of detected progesterone and testosterone for twenty-two samples processed by radioimmunoassay.

DISCUSSION

Commercial RIA kits offer 17β -estradiol, testosterone, and progesterone specific antibodies that identify the common structures present in humans. Here in the present study it was demonstrated that the required acceptance criteria for validated RIA parameters were fulfilled. This means that with adequate extraction procedures it is possible to quantify “vertebrate-type” sex steroid hormones (e.g., testosterone and progesterone) in octopus gonad extracts using components from commercially available RIA kits (i.e., ^{125}I -labeled hormone and coated tubes). This approach represents savings in both time and money, by eliminating time lost in screening animals potentially producing steroid antisera and the costs of maintaining them (Montes-Pérez & Pech-Martínez, 1998). It is widely acknowledged that sex steroid hormones are ubiquitous in all the animal kingdom and that they perhaps share the same common molecular formula with their vertebrate counterparts, although possibly with different structural homologies and functional analogies in distinct species (Sandor, 1980; Finch & Rose, 1995; Stoka, 1999; Lafont, 2000; Lafont & Mathieu, 2007).

Sex steroid hormones (e.g., progesterone and testosterone) in tissues of invertebrates are difficult to estimate and compare because of variations among species, organs, extraction procedures, and quantification methods (Lafont & Mathieu, 2007). Nevertheless, to our knowledge our study shows for the first time in *Octopus maya* a reliable radioimmunoassay

technique, fulfilling all recommended acceptability criteria (i.e., specificity, sensitivity, accuracy, precision, and reproducibility) (Skelley et al., 1973; Rodbard, 1974; Abraham, 1975; Cekan, 1975; Abraham et al., 1977; Hafs et al., 1977; Cekan, 1979; Chase, 1979; Bedolla-Tovar et al., 1984; IAEA, 1984; Ezan et al., 1991; Krotzky & Zehe, 1995; Alvarez-Cervera, 2002). Unfortunately, radioimmunoassay techniques used to quantify progesterone and testosterone in other octopuses such as *Eledone cirrhosa* (Nikitina et al., 1977; Young, 1992), *Octopus mimus* (Olivares-Paz et al., 2003), and *Octopus vulgaris* (D’Aniello et al., 1996; Di Cosmo et al., 2001) were not clear enough in their extraction procedure to be followed as a standardized technique. In other hands, some of these RIAs were developed using tritium (^3H) as tracer (D’Aniello et al., 1996; Di Cosmo et al., 2001). ^3H has a relatively low specific activity (1.07 PBq/mol) with respect to ^{125}I , but it is a radiation hazard when inhaled, ingested via food or water, or absorbed through the skin. Meanwhile, ^{125}I is preferred due to its higher specific activity (80.26 PBq/mol) and shorter half-life and, as a direct result, lower radioactivity with reduced hazards (AB, 2002).

On the other hand, only some acceptability criteria have previously been evaluated in octopus RIAs. The smallest amounts of hormones significantly different from zero detected (i.e., sensitivity) were 0.007 ng·g⁻¹ progesterone in *Octopus vulgaris* (Di Cosmo et al., 2001) and 0.005 ng·g⁻¹ in *Octopus mimus* (Olivares et al., 2003); while testosterone sensitivity has only been reported by Olivares et al. (2003) as

around $0.003 \text{ ng}\cdot\text{g}^{-1}$. In our study, sensitivities were $0.06 \text{ ng}\cdot\text{g}^{-1}$ for progesterone and $0.03 \text{ ng}\cdot\text{g}^{-1}$ for testosterone. But, on the basis of our results, it was evident that the changes in sensitivity (defined by the value of the slope of the standard curves) for both progesterone and testosterone did not vary more than 20% while the half-life of the ^{125}I elapsed. Moreover, all standard curves showed a strong positive correlation ($r = 0.99$) as demonstrated by the curve-fitting “logit-log” method; suggesting that the sensitivity of both RIAs is, therefore, relatively stable and acceptable throughout the entire ^{125}I half-life, and thus can be used with confidence during this period.

Our results thus indicate that precision and reproducibility were satisfactory in both hormone radioimmunoassays, where the value of %CV should not exceed $\pm 20\%$ (Abraham et al., 1977; Krotzky & Zeeh, 1995) or where the temporal stability of the system (i.e., ratio of “within-assay” to “between-assay” variance) must fall over 70% (Rodbard, 1974). Similarly, some coefficients of variation have been reported for hormone RIAs in other octopuses, such as *Octopus mimus* (progesterone, 5% average intra-assay; testosterone, 4% average intra-assay, Olivares et al., 2003) and *Octopus vulgaris* (progesterone, 8% intra-assay and 10% inter-assay, Di Cosmo et al., 2001). According to Rodbard (1974), Abraham (1975), Cekan (1975, 1979), Hafs et al. (1977), Bedoya-Tovar et al. (1984), IAEA (1984) and Ezan (1991) precision and reproducibility are the most essential components for hormone RIAs, and must be updated after each assay and when a process of sequential decision-making is used.

The range of *Octopus maya* gonad progesterone and testosterone concentrations quantified in this study encompasses the range found in other octopuses, such as *Eledone cirrhosa* (Nikitina et al., 1977; Young, 1992), *Octopus mimus* (Olivares-Paz et al., 2003), and *Octopus vulgaris* (D’Aniello et al., 1996; Di Cosmo et al., 2001). In the literature, there is little information on the percentage of recovered hormone. In fact, the great variability in progesterone and testosterone concentrations reported (Table 1) could be related to the solvent used to extract the hormones, the amount of solvent, and the number of extractions that were applied to recover the hormones from tissue (Abraham, 1975; Abraham et al., 1977). For example, Nikitina et al. (1977) performed two extractions

with acetone, D’Aniello et al. (1996), Olivares-Paz et al. (2003) performed two extractions with diethyl-ether, and Di Cosmo et al. (2001) performed one extraction with methanol. In our study it was observed that the amount of solvent used during extraction is a determining factor in the recovered concentration of hormone, since increasing the solvent amount (i.e., 15 to 30 mL of diethyl-ether per each extraction) also increased the amount of recovered hormone. Abraham (1975) and Abraham et al. (1977) reported this same effect.

The requirement for more than one extraction could be related to the lipid matrix in the tissue that commonly interferes with radioimmunoassay performance (Abraham, 1975; Abraham et al., 1977; Hafs et al., 1977; Rash et al., 1980). Octopus ovaries accumulate egg-yolk vitellins (i.e., the major yolk lipoproteins that serve as the nutritive material for development of the embryo and early larva) during maturation (O’Dor & Wells, 1973; Gnap, 1987; Pollero & Iribarne, 1988; Boyle & Chevis, 1992; Rosa et al., 2004; Siero et al., 2006, among many others); therefore, this condition should be taken into account, particularly in females, when hormones are being extracted. This problem could be solved by using more than two extractions, as was observed here.

An extremely important factor in determining the magnitude of the estimated concentration of hormones in a gonad extract is the nature of the method by which the hormonal concentrations are measured. It has been reported that precise and accurate assays such as RIA do not show identical results with other extremely sensitive, accurate and precise analytical methods such as HPLC-EIA (Van de Wiel & Koops, 1986) and LC-MS/MS (Gust et al., 2010). Several authors (Abraham, 1975; Cekan, 1975, 1979; IAEA, 1984; Srikandakumar et al., 1986; Ezan et al., 1991; Chard, 1995; Montes-Pérez, 1995; Alvarez-Cervera, 2002, among many others) have indicated that the amount of quantified hormone is highly variable according to: (1) immunoassay type (liquid-phase or solid-phase); (2) type of label on the tracer (Iodine or Tritium); (3) sample treatment method (direct or indirect); (4) antibody specificity; and (5) type of standards (commercial or elaborated).

Therefore, the reported concentrations of hormones as determined by RIA correspond to relative magnitudes, which are reliable according to the used method. In any case, the

meaning of the considered value is not only based on the criteria which the method was validates, but is also based on the consistency and validity of the method itself and the congruence of these values with the biological process being studied, rather than by “confirmative proofs” with other analytical methods (Montes-Pérez, 1995; Escrig-Sos et al., 2006; Gust et al., 2010).

In summary, it can be concluded that the developed and validated RIAs meet the requirements for the quantification of progesterone and testosterone in *Octopus* gonad extracts and show that it is possible to use ^{125}I -labeled hormone and plastic coated-tubes from commercial RIA kits. Therefore it is possible to use the procedures of these RIAs to analyse variations in hormone levels associated with gonad development stages and the reproductive cycle of octopuses, enabling investigations of their possible functions.

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